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Reduction of Synaptojanin1 Ameliorates Synaptic and Behavioral Impairments in a Mouse Model of Alzheimer's Disease

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Abstract

Decades of research have correlated increased levels of amyloid β-peptide (Aβ) with neuropathological progression in Alzheimer's disease (AD) patients and transgenic models. Aβ precipitates synaptic and neuronal anomaliesby perturbing intracellular signaling which, in turn, may underlie cognitive impairment. Aβ also alters lipid metabolism, notably causinga deficiency of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$], a phospholipid that regulates critical neuronal functions. Haploinsufficiency of the gene encoding synaptojanin 1 (Synj1), a major $PI(4,5)P_2$ phosphatase in the brain, provided protection against $PI(4,5)P_2$ breakdown and electrophysiological deficits attributable to Aβ. Based on these data, we tested whether reduction of Synj1 could rescue cognitive deficits and Aβ-induced morphological alterations of synapses. We found that hemizygous deletion of *Synj1* in the context of a mouse model expressing the Swedish mutant of amyloid precursor protein rescues deficits in learning and memory without affecting amyloid load. *Synj1* heterozygosity also rescued $PI(4,5)P_2$ deficiency in a synaptosome-enriched fraction from the brain of Tg2576 mice. Genetic disruption of *Synj1* attenuated Aβ oligomer-induced changes in dendritic spines of cultured hippocampal neurons, sparing maturespine classes, which corroborates the protective role for Synj1 reduction against Aβ insult at the synapse. These results indicate that Synj1 reduction ameliorates AD-associated behavioral and synaptic deficits, providing evidence that Synj1 and, more generally, phosphoinositide metabolism, may be promising therapeutic targets. Our work expands on recent studies identifying lipid metabolism and lipid modifying enzymes as targets of AD-associated synaptic and behavioral impairment.

Introduction

A pathogenic hallmark of Alzheimer's disease (AD) is the cleavage of amyloid precursor protein (APP) leading to accumulation of amyloid β-peptide (Aβ) and subsequent deposition in plaques (Bertram and Tanzi, 2012). Accumulation of soluble Aβ oligomers closely correlates with cognitive decline and disease progression in animal models and AD patients primarily through disruption of synaptic plasticity (Benilova et al., 2012). Soluble Aβ oligomers also profoundly alter dendritic spine morphology in dissociated cultures,

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hippocampal slices and in animal models of AD (Pozueta et al., 2012). While these synaptic defects can be modulated by altering glutamatergic receptor trafficking and actin cytoskeleton dynamics (Pozueta et al., 2012), the molecular mechanisms orchestrating downstream effects of Aβ oligomers remain poorly understood.

Recent work has indicated that perturbation ofphosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] may be relevant to the synaptotoxic actions of Aβ oligomers and more generally to AD (Landman et al., 2006; Berman et al., 2008; Di Paolo and Kim, 2011). The catabolism of $PI(4,5)P_2$, a signaling lipid critical for ion channel regulation, exo/endocytosis, actin cytoskeleton rearrangement and signal transduction, is tightly controlled by the polyphosphoinositide phosphatase, synaptojanin1 (Synj1) (Di Paolo and De Camilli, 2006). Synj1 plays a critical role in synaptic vesicle trafficking, actin dynamics and AMPAR internalization (Di Paolo and De Camilli, 2006; Gong and De Camilli, 2008). Neurons derived from mice deficient in $PI(4,5)P_2$ dephosphorylation due to haploinsufficiency of the gene encoding Synj1 (*Synj1*) display resistance to Aβ42 oligomer effectson $PI(4,5)P_2$ destabilization and electrophysiological synaptic impairment (c). Recent work has further connected phosphoinositide metabolism to AD by identification of the *Synj1* ortholog, *INP52*, in anunbiased genome-wide screen for Aβ toxicity modifiers in yeast (Treusch et al. 2011). Further, *Synj1* displayed transcriptional changes in a mouse model of AD and in AD patients (Miller et al., 2008; Alldred et al., 2012). Altogether, these studies suggest that $PI(4,5)P_2$ imbalance may play a critical role in AD pathogenesis.

In this study, we show that *Synj1* haploinsufficiency protects against memory impairment in an AD mouse model, Tg2576 (Hsiao, 1996). To address the underlying cause of this behavioral rescue, we examined morphological changes in dendritic spines triggered by Aβ oligomers in cultured neurons and found that spine alterations were attenuated by reduction of Synj1.

Materials & Methods

Mouse models

Synj1$^{+/-}$ mice (Cremona et al., 1999) were bred to Tg2576 (Hsiao et al., 1996, Taconic) to create Tg2576/*Synj1* F1 progeny. Since *Synj1*$^{-/-}$ mice do not survive to adulthood, *Synj1*$^{+/-}$ mice were used, giving rise to four genotypes:*Synj1*$^{+/+}$, *Synj1*$^{+/-}$, Tg2576/*Synj1*$^{+/+}$and Tg2576/*Synj1*$^{+/-}$. Both sexes were used for experiments, but no gender specific defects were found.

Fear conditioning (FC)

Contextual FC is a hippocampus/amygdala-dependent task in which Tg2576 mice show deficits (Barnes et al., 2005). The test was performed on 5–6 month old mice as previously described and cued FC, a hippocampus-independent task, was used as a control (Oliveira et al., 2010).

Two-day radial arm water maze (2-day RAWM)

SinceTg2576 mice show deficits in reference memory in the *2-day RAWM*, 7–8 month old mice were tested as previously described (Alamed et al, 2006).

Novel object recognition task (NOR)

Tg2576 mice show deficits in the NOR memory task (Hernandez et al., 2010). Littermates at 5–6 months of age were tested in NOR and onset of the exploration time was defined as the moment the head of the animal approached the object within a 2.5cm radius.

Biochemistry

Western analysis was accomplished using antibodies to Synj1 (Haffner et al., 1997), neuronal β-tubulin (TUJ1, Covance)and human APP (6E10, Covance). Synj1 was quantified using Fugifilm LAS3000 and MultiGaugev3.0 imaging software and APP was quantified using Licor Odyssey infrared detection. For quantification of Aβ40 and Aβ42 (ELISA, Invitrogen) brain tissue was processed as previously described (Schmidt et al., 2005).

Lipid analysis

To quantify phosphoinositide levels in brain tissue, HPLC combined with suppressed conductivity detection was used (Berman, et al., 2008). The crude synaptosome fraction (P2) was isolated from brains prior to lipid extraction (Wu, et al., 1986).

Dendritic spine analysis in cultured primary neurons

Hippocampal primary cultures were prepared from neonatal pups (Berman et al., 2008) and cultured for 21 days. Cultures were exposed to 200nM Aβ oligomer for 24 hours, fixed with 4% paraformaldehyde and DiOlistically labeled with Dil (Molecular Probes, Smith et al., 2009). Images were collected with 100x objective and 3.14x zoom using a z-stack of 0.3μm sections on a Nikon C1 digital confocal system attached to an Olympus IX71 inverted scope. Neuron Studio (Rodriguez et al., 2008) was used to analyze spine density and spine class.

Statistical methods

For analysis of behavioral tests, one-way ANOVA was used with Tukey's Multiple Comparison Test except for FC data, which were not normally distributed. The non-parametric Kruskal-Wallis test was used to analyze contextual FC data ($p = 0.056$). There were no significant differences between the groups at baseline, pre-cue or post-cue freezing using the Kruskal-Wallis test. In order to better fit the normality assumptions of ANOVA, the contextual FC data were transformed using Log_{10} and re-analyzed using one-way ANOVA ($p = 0.045$). One-way ANOVA and Tukey's Multiple Comparison Test were used for the phospholipid analysis. Student's t-test was used for all other biochemical experiments and spine analysis using 2-tailed distribution with equal variance ($p < 0.05$). All data are shown as geometric mean ± SEM.

Results

Cognitive rescue in Tg2576/*Synj1*$^{+/-}$ mice

To investigate the effects of hemizygous deletion of *Synj1* on learning and memory impairment, we employed a battery of three behavioral tests, contextual FC, two-day RAWM and NOR. In contextual FC, a hippocampus- and amygdala-dependent learning task (Maren, 2008), Tg2576 mice exhibit deficits at 5–6 months of age which precedes amyloid plaque deposition (Hsiao et al., 1996; Oliveira et al., 2010). While Tg2576/*Synj1*$^{+/+}$ showed decreased freezing and thus impaired contextual fear memory when exposed to the conditioning context 24h after training, Tg2576/*Synj1*$^{+/-}$ mice showed normal freezing as their wild type (WT) and *Synj1*$^{+/-}$ littermates (Fig. 1A). In contrast to contextual FC, auditory cued FC, a hippocampus-independent task, showed no differences among genotypes (Fig. 1B).

The2-day RAWM taske valuates reference memory by scoring entry into a maze arm without the escape platform as an error. Tg2576 mice typically show impaired ability to learn which arm of the maze has the escape platform (Alamed et al., 2006). Accordingly, Tg2576/*Synj1*$^{+/+}$ mice did not effectively learn to locate the escape platform. In contrast, WT, *Synj1*$^{+/-}$, and importantly, Tg2576/Synj1$^{+/-}$ mice showed a progressive decrease in

errors over the course of the experiment, indicating that they had learned the task (Fig. 1C). The visible platform task was used to evaluate potential baseline differences in sensory or motor performance or motivation between genotypes. Some Tg2576/*Synj1*$^{+/+}$ mice avoided the visible platform due to neophobia (session 2–3), but the difference in latency was abolished by trial 4 (Fig. 1D, Alamed et al., 2006). Additionally, there were no consistent differences in the mouse swim speed (Fig. 1E).

Published studies have shown that Tg2576 mice exhibit NOR deficits (Hernandez et al., 2010). All mice explored both objects equally on the training day (Fig. 1F). As expected, on the testing day (Fig. 1G), Tg2576/*Synj1*$^{+/+}$ mice spent the same amount of time with each object indicating they were unable to discriminate between the novel and original objects. Tg2576/*Synj1*$^{+/-}$ animals, however, displayed the same exploratory behavior as WT and *Synj1*$^{+/-}$ mice, spending more time with the novel object. Consistent with published results (Gil-Bea et al., 2007), we observed hyperlocomotor activity of Tg2576 mice in the open field test of NOR testing and this phenotype was rescued in the Tg2576/*Synj1*$^{+/-}$ mice (Fig. 1H). Thus, three separate behavioral tests revealed that reduction of *Synj1* in Tg2576 mice was sufficient to ameliorate deficits in learning and memory.

Effect of hemizygous deletion of *Synj1* on Aβand phosphoinositide levels in a mouse model of AD

We investigated the possibility that haploinsufficiency of *Synj1* may affect Aβ and phosphoinositide levels in the AD model. Western blot analysis showed no differences in the levels of the transgene, human APP_{sw}, in Tg2576/*Synj1*$^{+/+}$ and Tg2576/*Synj1*$^{+/-}$ mice (Fig. 2A, B). In both the haploinsufficient genotypes, *Synj1*$^{+/-}$ and Tg2576/*Synj1*$^{+/-}$, Synj1 protein levels were reduced compared to*Synj1*$^{+/+}$ and Tg2576/*Synj1*$^{+/+}$ genotypes (Fig. 2A, C). Neither Aβ42 nor Aβ40 levels were affected by haploinsufficiency of *Synj1* (Fig. 2D). Importantly, the levels of $PI(4,5)P_2$, but not control inositol lipids phosphatidylinositol (PI) and phosphatidylinositol-4-phosphate (PI4P), were significantly reduced in the synaptosome-enriched (P2) fraction from forebrain tissue of Tg2576mice and restored to wild-type levels in Tg2576/*Synj1*$^{+/-}$ mice (Fig. 2E). Interestingly, these changes were observed in synaptosome-enriched fractions, but not in whole forebrain extracts (data not shown), suggesting that alterations in $PI(4,5)P_2$ in the Tg2576 model may besynapse-specific.

Effect of Synj1 reduction on Aβ-induced changes in dendritic spine morphology

To investigate the underlying cause of the observed behavioral rescue due to reduced Synj1 and $PI(4,5)P_2$ maintenance, we investigated the effects of *Synj1* reductionon structural spine modifications caused by soluble Aβ oligomers. Dendritic spines, which are protrusions on dendritic processes of excitatory neurons, undergo dynamic structural changes that are closely associated with learning and memory (Bosch and Hayashi, 2012) and are profoundly affected by Aβ oligomers (Pozueta et al., 2012). We analyzed spine morphology in dissociated hippocampal primary cultures of *Synj1*$^{+/+}$, *Synj1*$^{+/-}$or *Synj1*$^{-/-}$ mice. Under basal conditions, density and head diameter were not altered among the genotypes though in *Synj1*$^{+/-}$ and*Synj1*$^{-/-}$ neurons, dendritic spines were significantly longer (Fig. 3A–D). Prior studies have reported decreased spine density and increased spine length after treatment of cultured neurons with Aβ oligomers (Calabrese et al., 2007; Lacor et al., 2007), both of which were recapitulated in wild type neurons treated with Aβ (Fig. 3A–C). However, in *Synj1*$^{+/-}$ and *Synj1*$^{-/-}$ neurons, density was preserved and spine length did not increase following treatment with Aβ oligomers (Fig. 3A–C). When spines were analyzed by class, we observed stubby and thin spines in *Synj1*$^{+/-}$ and *Synj1*$^{-/-}$neurons were selectively spared from Aβ oligomer-induced decrease in density (Fig. 3A, E). Furthermore, in *Synj1*$^{-/-}$ neurons, mushroom spines were spared in addition to thin and stubby spines (Fig. 3A, E).

These results support the hypothesis that neuronswith reduced levels of Synj1 are fortified against synaptic defects induced by Aβ oligomers.

Discussion

We previously identified PI(4,5)P_2 metabolism as a target of familial AD-linked presenilin mutations and Aβ oligomers (Landman et al., 2006; Berman et al., 2008; Di Paolo and Kim, 2011). Haploinsufficiencyof *Synj1* was found to cause a decrease in the dephosphorylation of brain PI(4,5)P_2 (Voronov et al., 2008) and conferred protection against Aβ-induced defects in long-term potentiation (Berman et al., 2008). In this study, we investigated the role Synj1 reduction plays in behavioral deficits in a mouse model of A Das well as morphological alterations in dendritic spines triggered by Aβ oligomers. We found that *Synj1* haploinsufficiency was sufficient to ameliorate learning and memory deficits in the Tg2576mouse model of AD in three independent behavioral paradigms, contextual FC, RAWM and NOR. Because Tg2576 animals do not develop extensive plaque pathology in the age group we studied (5–9 months), their learning deficits are likely due to the accumulation of soluble pools of Aβ (Hsiao et al., 1996). However, Aβ levels were unaltered by the reduction of Synj1 suggesting that the protective effects on behavior are independent of Aβ levels in Tg2576/*Synj1*$^{+/-}$ mice, likely reflecting interference with Aβ-induced synaptotoxic signaling. Because our previous work showed Aβ42 oligomers cause a decrease in PI(4,5)P_2, the major substrate for Synj1 (Berman et al, 2008), we investigated PI(4,5)P_2metabolism in our *in vivo* models. Though we found no global reduction of PI(4,5)P_2 levels in whole forebrain extracts, synaptosome-enriched (P2) fractions displayed reduced PI(4,5)P_2 levels in Tg2576 mice but notinTg2576/*Synj1*$^{+/-}$ mice. This supports the hypothesis that synaptic pools of PI(4,5)P_2 are affected by Aβ and preserved by the absence of one copy of *Synj1* in this mouse model. Interestingly, despite reports indicating Aβ biogenesis and APP processing are modulated by PI(4,5)P_2 (Landman et al., 2006; Osawa et al., 2008; Osenowski et al., 2008), we found no alterations in full-length APP, Aβ40 or Aβ42levels in the Tg2576/*Synj1*$^{+/-}$ cross, suggesting that specific pools of PI(4,5)P_2involved in APP processing may not be affected by the partial reduction of Synj1 in the context of APP_{sw} overexpression.

To understand the cellular mechanism underlying the protective role of Synj1 deficiency, we investigated changes in spine morphology after Aβ insult. Cultured hippocampal neurons with reduced Synj1 maintained normal spine density, length and mature classes of spines, namely mushroom, thin and stubby, in the presence of Aβ oligomer. One possible mechanism underlying maintenance of spine morphology in the presence of Aβ challenge, as well as amelioration of the learning and memory deficits, is regulation of synaptic glutamate receptor trafficking which has been shown to affect spine morphology under non-pathological conditions (Kopec et al., 2006) and to be sensitive to Aβ (Pozueta et al., 2012). Importantly, a recent study has demonstrated that ablation of*Synj1* delays AMPA receptor (AMPAR) internalization (Gong and De Camilli, 2008). Reduction of Synj1 and resulting PI(4,5)P_2 maintenance may thus delay the loss of AMPAR from the synaptic membrane due to Aβ challenge, thereby preserving spine morphology and synaptic function. It has also been reported that the NMDA receptor (NMDAR) interacts with PI(4,5)P_2 through α-actinin, an actin crosslinking protein (Michailidis et al., 2007), and PI(4,5)P_2-mediated regulation of NMDAR trafficking is impaired by Aβ challenge (Mandal and Yan, 2009). Since Aβ has been reported to decrease NMDAR surface expression (Pozueta et al., 2012), reduction of Synj1 could prevent this phenomenon through maintenance of PI(4,5)P_2resulting in more persistent NMDAR signaling. Synj1 may also have a role downstream of Aβ signaling as a substrate of calcineurin, a Ca^{2+}-activated phosphatase that controls synaptic plasticity and has been shown to stimulate Synj1 via dephosphorylation (Lee et al., 2004). Indeed, calcineurinsignaling has been shown to mediate Aβ-induced spine

loss (Shankar et al., 2007), raising the possibility that a relevant target of this phosphatase downstream of Aβmay be Synj1. Finally, reduced Synj1 levels may maintain synaptic pools of $PI(4,5)P_2$ preventing actin destabilization, which has been previously reported to occur in response to Aβ oligomer treatment (Shankar et al., 2007).

Overall, our studies validate Synj1 at the genetic level as a candidate therapeutic target for AD. Since Aβ-lowering strategies targeting late-stage AD have had only modest success in recent clinical trials (Huang and Mucke, 2012), identification of alternate targets that are independent of amyloid load, such as Synj1, is critical for progress in the development of AD therapeutics. Our data revealed that heterozygous deletion of the Synj1 gene ameliorates AD-associated cognitive deficits and protects synapses against Aβ, without interfering with normal behavior or synaptic function. Thus, reducingSynj1 levels may give rise to the desired protective phenotype without interfering with normal brain function in AD patients. Reducing Synj1 activity may also be beneficial in ameliorating the cognitive deficits of Down syndrome, characterized by over expression of genes on chromosome 21, including *Synj1* and *App*, and inevitably leading to AD pathology in adulthood (Voronov et al., 2008 and Cossec et al., 2012). Confluent with a validated therapeutic target for diabetes, phosphoinositide phosphatases represent a new and promising class of therapeutic targets in human diseases (McCrea and De Camilli, 2009). In addition to phosphatases, a phosphoinositide kinase has also been implicated in AD pathology. Inhibition ofPI3-kinase has been demonstrated to decrease Aβ biogenesis (Petanceska et al., 1999 and Haugabook et al. 2001) and ameliorate AD-associated cognitive defects (Chiang et al., 2010). Finally, recent work has shown that genetic disruption of phospholipases, such as phospholipase D2 and cytosolic phospholipase A2, improves pathological phenotypes in AD mouse models (Oliveira et al., 2010; Sanchez-Mejia et al., 2010; and Chan et al., 2012). Collectively, these studies substantiate that modulation of neuronal lipid networks can ameliorate AD-associated pathologies and therefore targeting lipid-modifying enzymes represents an engaging strategy for future development of AD therapeutics.

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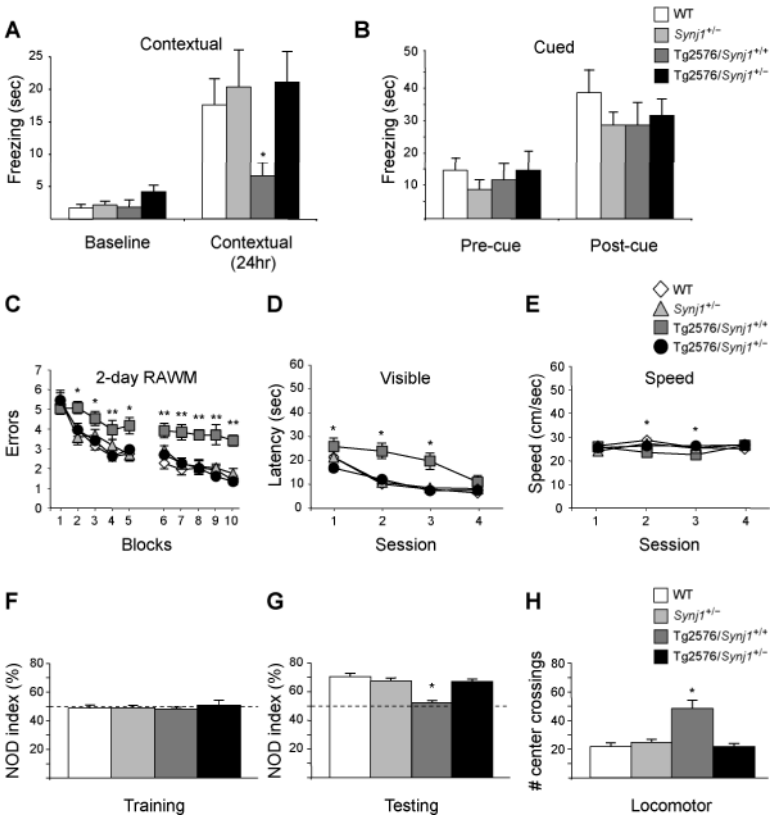

Fig. 1.

Hemizygous deletion of *Synj1* ameliorates memory and behavioral deficits in Tg2576 mice. **A**, Freezing response in the FC paradigm in 5–6 month old WT (n=12), $Synj1^{+/-}$ (n=16), Tg2576/$Synj^{+/+}$ (n=8), and Tg2576/$Synj1^{+/-}$ (n=12) mice. **B**, Freezing responses in the auditory cued FC with the mice used in (A) **C**, Two-day RAWM. WT (n=9), $Synj1^{+/-}$ (n=9), Tg2576/$Synj1^{+/+}$ (n=8) and Tg2576/$Synj1^{+/-}$ (n=9) **D**, Visible platform test; WT (n=9), $Synj1^{+/-}$ (n=9), Tg2576/$Synj1^{+/+}$ (n=7) and Tg2576/$Synj1^{+/-}$ (n=9). **E**, Swim speed;WT (n=9), $Synj1^{+/-}$ (n=9), Tg2576/$Synj1^{+/+}$ (n=7) and Tg2576/$Synj1^{+/-}$ (n=9). **F**, NOR training; WT (n=9), $Synj1^{+/-}$ (n=9), Tg2576/$Synj1^{+/+}$ (n=7), and Tg2576/$Synj1^{+/-}$ (n=9). Exploration time is expressed by Novel Object Discrimination (NOD) Index = amount of time spent exploring novel object *100 / time exploring novel object + time exploring familiar object. **G**, NOR testing. **H**, The hyperlocomotor activity displayed by mice tested in NOR.

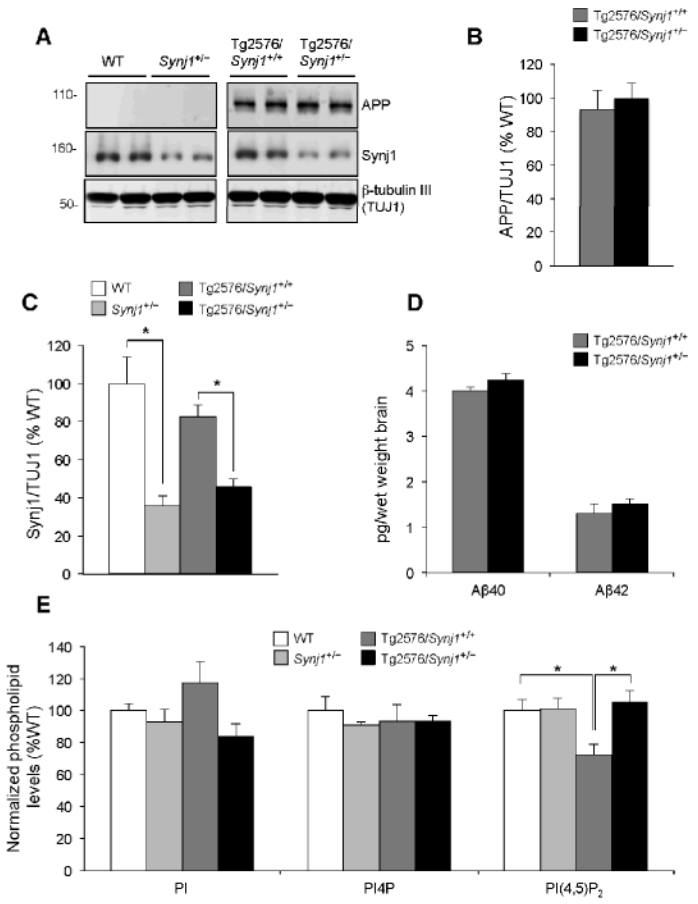

Fig. 2.

APP and Aβ levels are not significantly altered by reduction of Synj1 in Tg2576 mice. **A**, Western blot detection of human APP (6E10), Synj1 and anti-neuronal β-tubulin (TUJ1) from brain of WT (n=6), *Synj1*$^{+/-}$ (n=7), Tg2576/*Synj1*$^{+/+}$ (n=5) and Tg2576/*Synj1*$^{+/-}$ (n=7) mice. **B**, APP levels as normalized to TUJ1. **C**, Synj1 levels as normalized to TUJ1. **D**, Detection of Aβ42 and Aβ40 from brain of Tg2576/*Synj1*$^{+/+}$ (n=5) and Tg2576/*Synj1*$^{+/-}$ (n=7) mice. **E**, Inositol lipid levels from synaptosome-enriched (P2) fractions derived from forebrain tissue of WT (n=9), *Synj1*$^{+/-}$ (n=6), Tg2576/*Synj1*$^{+/+}$ (n=6) and Tg2576/*Synj1*$^{+/-}$ (n=6) mice, as determined by HPLC combined with suppressed conductivity.

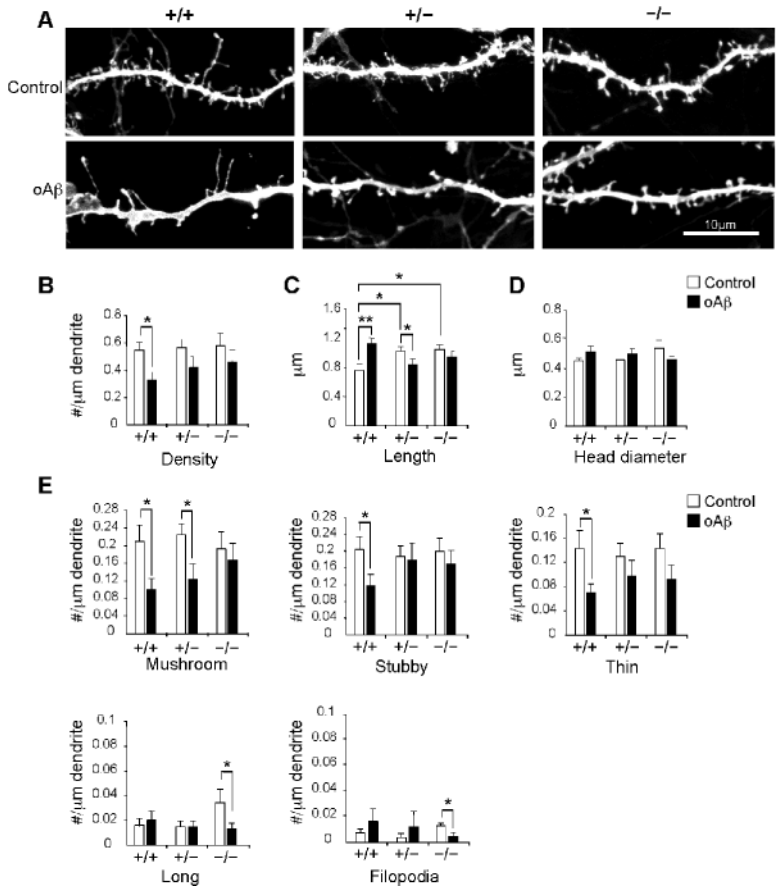

Fig. 3.

Alterations of spine morphology attributed to Aβ are ameliorated with reduction of Synj1. **A**, Dendritic spines in hippocampal neuronal neuronsexposed to 200nM Aβ oligomer (o Aβ) for 24 hours and DiOlistically labeled from WT(+/+) cultures (n=4): DMSO treated (n=22) and o Aβ treated (n=21); *Synj1*$^{+/-}$ (+/−) cultures(n=4):DMSO treated (n=22) and o Aβ treated (n=20); or *Synj1*$^{-/-}$ (−/−) cultures(n=3):DMSO treated (n=19) and o Aβ treated (n=19); Bar 10μm. **B**, Spine density. **C**, Spine length. **D**, Spine head diameter. **E**, Spine class analysis using Neuron Studio which distinguishes spine classes on basis of length and presence of a spine head. Mushroom, stubby and thin are less than 2μm while long spines and filopodiaare more than 2μm long. Mushroom and long spines have a head, while the other classes do not. The total number of spines analyzed was 3,745 for 7,415μm length of dendriteas follows: *Synj1*$^{+/+}$ (DMSO: 758; oAβ: 414); *Synj1*$^{+/-}$ (DMSO: 722; oAβ: 408); *Synj1*$^{-/-}$ (DMSO: 686; Aβ: 757).